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The endothelium in sepsis

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CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE THESIS

1.1 GENERAL INTRODUCTION

1.1.1 GENERAL ASPECTS OF SEPSIS

Sepsis describes a complex clinical syndrome, which is characterized by a systemic inflammatory response of the organism to the presence of an infection ¹. Due to their impaired immune system, the very young and very old are particularly prone to develop sepsis. Furthermore, persons of all ages are at risk, who are recovering from major surgery or trauma, who are receiving immuno-modulating drugs or total parenteral nutrition, or who have immuno-compromising diseases (e.g., cancer, diabetes, or AIDS). The severity of sepsis varies widely. Although in some patients the inflammatory response may be relatively mild and self-limited, in many patients sepsis may lead to organ dysfunction, organ hypoperfusion and hypotension. Septic shock occurs in a subset of patients suffering from severe sepsis and refers to a condition in which hypotension does not respond to fluid resuscitation.

Sepsis is a leading cause of death among the elderly, especially on the Intensive Care Unit. According to surveys of the German Sepsis-Network “SepNet”, about 300 per 100.000 people contract sepsis each year in Germany. This is comparable to the incidence of myocardial infarction (360/100.000), and clearly higher than the incidence of breast cancer (110/100.000) and of colon cancer (50/100.000). Findings in the United States of America, representative for industrialized countries, showed identical results (3 cases per 1.000 population) ². When looking at the general causes of death, sepsis has a death rate of 9% and therefore a similar importance as myocardial infarction ³. In-hospital mortality among patients with severe sepsis was recently estimated to be 29%, although rates as high as 50% have been reported in the literature ^{2,4}. For patients with septic shock, mortality is even higher ^{5,6}. Not surprisingly, the economic costs of hospital care for patients with severe sepsis are substantial, >23,000 EUR per patient in Germany (\$ 22.100 in the United States), which makes it one of the most cost-intensive diseases of our time ^{2,3}. Despite considerable effort to understand the systemic inflammatory response and characteristics of severe sepsis and despite the progress in critical care management, no significant improvement in the mortality of septic patients could be noticed over the past decade ^{7,8}.

1.1.2. PATHOGENESIS AND PATHOPHYSIOLOGY OF SEPSIS

The causes of sepsis are multifactorial and can include practically any infectious organism. For a long time, infections with Gram-negative bacteria have been considered to be the most frequent cause of this condition ⁹. Recent studies however, have indicated

that Gram-positive infections are more frequently found in septic patients ⁸. An effective immune response to pathogens depends on the proper activation, regulation and effector function of immune cells. The primary function of innate immunity is to recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, which trigger signals resulting in pro-inflammatory gene expression, leukocyte chemotaxis, phagocytosis, cytotoxicity and activation of adaptive immune responses ¹⁰. To restore the equilibrium, an anti-inflammatory response is induced subsequently. In a favorable case, homeostasis is re-established. However, an overreaction of the pro-inflammatory response can lead to a compensatory anti-inflammatory response in which the pro-inflammatory and anti-inflammatory responses counterbalance each other. The body is in a state of immune paralysis and unable to produce an adequate immune response ¹¹.

To understand the pathophysiology of sepsis, we have to bear in mind that it comprises multiple derangements involving several different organs and systems. In general, sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated, e.g., septic patients have substantial, life-threatening alterations in their coagulation system. In the past, it has been suggested that sepsis merely represents an excessive hyper-inflammatory response with patients dying from inflammation-induced organ injury. More recent data indicate that considerable heterogeneity exists in the inflammatory response of septic patients. Some patients appear to be immuno-stimulated, since increased levels of pro-inflammatory cytokines can be detected in the peripheral blood ¹², whereas others appear suppressed and show decreased levels of pro-inflammatory cytokines ¹³. Heterogeneity is also found in the cellular changes. The function of some cells are enhanced such as neutrophils that remain activated for an extended time. Other cellular changes become accelerated in a destructive way including lymphocyte apoptosis. Furthermore, metabolic changes are observed in septic patients ^{14, 15}. To present knowledge no single mediator/system/pathway/pathogen drives the pathophysiology of sepsis, implying a highly complex pathology ¹⁶.

Inflammatory Response

The inflammatory response represents an important element of the immune response to pathogens, because an appropriate inflammatory response eliminates the invading microorganisms without causing too much damage to tissues or organs.

During the onset of sepsis the inflammatory system becomes hyperactive and an array of pro-inflammatory mediators are released, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-8. The primary role of these cytokines is to enhance leukocyte infiltration into sub-endothelial compartments at the site of infection to control and clear the invading pathogen rapidly. Simultaneously, strong production of

acute-phase-proteins, such as C-reactive protein, occurs and the complement system is activated.

Much attention has been given to the relevance of the pro-inflammatory response in the clinical outcome of septic patients. This was based on the observation that septic patients with increased serum-levels of specific mediators such as TNF- α are at increased risk for death¹⁷. In animal studies, injection of TNF- α molecules resulted in widespread inflammatory alterations¹⁸ and tissue injury¹⁹ similar to that observed in septic patients. Furthermore, injection of lethal doses of the endotoxin lipopolysaccharide (LPS) resulted in elevated serum-levels of the same mediators and inhibition of these specific mediators improved survival in murine endotoxin shock models²⁰. These observations launched a series of clinical trials aimed at blocking TNF- α or IL-1 β , but these trials failed to show improvements in sepsis survival²¹⁻²³. One confounding factor is that TNF- α levels were not always increased in patients, in part because of the rapid kinetics of the TNF- α response^{24, 25}. Serum TNF- α and IL-1 β reach toxic levels in mice and human volunteers within 1-2 h after LPS infusion (fig. 1)^{26, 27}, and delayed treatment with anti-TNF- α or anti IL-1 β failed to prevent late endotoxin deaths^{28, 29}. Moreover, endotoxin-responsive mice treated with lethal doses of endotoxin often succumbed at latencies of up to 5 d, long after serum TNF- α and IL-1 β have returned to basal levels. The insights gained from the failure of clinical trials of anti-TNF- α strategies, as well as the observations regarding the kinetics of TNF- α release compared with the onset of lethality, prompted a search for a late mediator of endotoxin-induced death. In this search high-mobility group box (HMGB)-1 was identified as a late mediator of endotoxin lethality in mice³⁰. Originally, HMGB-1 was identified as a nuclear DNA-binding protein that functions as a structural cofactor for proper DNA-transcriptional regulation and gene expression³¹. Recent studies indicate that immune cells can liberate HMGB-1 into the extracellular milieu where it functions as a pro-inflammatory cytokine^{30, 32, 33}. Furthermore, HMGB-1 is recognized by the innate immune system as a necrotic marker to signal tissue damage^{34, 35}.

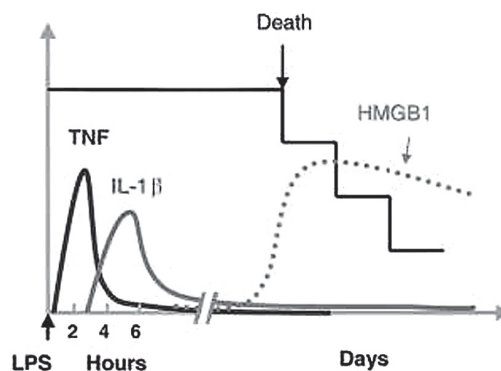


Figure 1. Early versus late mediators of endotoxin lethality in mice. Mice treated with a lethal dose of LPS succumb at latencies of up to several days, long after serum TNF- α and IL-1 β have returned to basal levels. HMGB-1 release occurs at a delayed stage of sepsis and parallels the onset of lethality. Reprinted with permission from Wang H, Yang H, Czura CJ, Sama AE, Tracey KJ. (2001) HMGB1 as a late mediator of lethal systemic inflammation. *Am J Respir Crit Care Med*;164:1768-73. Official Journal of the American Thoracic Society. © American Thoracic Society.

In a mouse model, serum HMGB-1 was minimally detectable at 8h after administration of a median lethal dose (LD₅₀) of LPS, and increased to a prolonged plateau level from 16 to 32 h after LPS treatment (fig.1) ³⁰. Delayed administration of antibodies to HMGB-1 attenuated endotoxin lethality in mice, and administration of HMGB-1 itself was lethal ³⁰. These characteristics distinguishes HMGB-1 from previously described early cytokine mediators of LPS lethality. In view of the late and prolonged kinetics of HMGB-1 in mice and considering that clinical signs of sepsis typically develop long after the early cytokine response to the acute infection, these results support the assumption that HMGB-1 might play a role in sepsis. In fact, in septic patients increased levels of HMGB-1 could be detected, whereas it was not detectable in healthy controls. In addition, serum HMGB-1 levels were significantly higher in septic patients who did not survive as compared with survivors ³⁰.

Another observation revealed that stimulation of the vagus nerve could attenuate production of inflammatory cytokines such as TNF- α ³⁶. The effect was mediated through the $\alpha 7$ subunit of the nicotinic acetyl choline receptor ³⁷, providing an innovative prospect for inhibition of the inflammatory cascade. Recently, transcutaneous vagus nerve stimulation was shown to not only reduce serum TNF- α levels, but also to inhibit serum HMGB-1 levels and improve survival in a murine sepsis model ³⁸. Whether this activity can be therapeutically utilized for septic patients has still to be proven.

The excessive pro-inflammatory response that occurs in sepsis is balanced by an array of counter-regulatory anti-inflammatory mediators that attempt to restore immunological equilibrium. Counter-inflammatory mediators include antagonists such as the soluble TNF receptors and IL-1 receptor antagonists, inactivators of the complement cascade and anti-inflammatory cytokines like IL-10, which lead to a decline in the production of many of the pro-inflammatory mediators. This is accompanied by a decrease in HLA-DR expression on monocytes ³⁹. Another aspect of down-regulation of immunity in the course of sepsis is the development of lymphocyte apoptosis. Extensive lymphocyte apoptosis is seen in animal models of sepsis and also occurs in septic patients, but much less in critically ill non-septic controls ^{40, 41}. Septic patients are usually lymphopenic, and analysis of autopsy tissue samples has demonstrated a selective depletion of B and CD4⁺ lymphocytes ⁴². The functional consequences of this comprise a more general immune

paralysis, characterized by T-cell hyporesponsiveness and anergy, a state of non-responsiveness to antigens. This counter-balancing response to the initial pro-inflammatory state can also be considered as an overresponse and an inadequate host defense against infection, therefore constituting a potential mediator of severe sepsis and progressive organ failure. This notion has prompted efforts to restore immune activation with agents such as interferon (IFN)- γ ³⁹, granulocyte colony-stimulating factor (G-CSF)⁴³ or granulocyte macrophage colony-stimulating factor (GM-CSF)⁴⁴. These clinical trials only showed little success in terms of improved survival⁴⁵. Taken together, these data indicate that the inflammatory response in septic patients is complex and multifactorial and not simply defined as enhanced or decreased. It rather displays a dynamic process and varies in time. While in the onset of sepsis the inflammatory system becomes hyperactive, in later stages the excessive pro-inflammatory response is counterbalanced by an anti-inflammatory response possibly resulting in immune paralysis (fig. 2). This was supported by the finding that PBMCs obtained from patients in earlier stages of sepsis, i.e., without organ dysfunction or shock, were hyperresponsive to LPS regarding production of inflammatory cytokines such as IL-6 and TNF- α ⁴⁶. And in contrast to this, patients with severe sepsis and septic shock displayed a down-regulation of pro-inflammatory cytokines in whole blood⁴⁷.

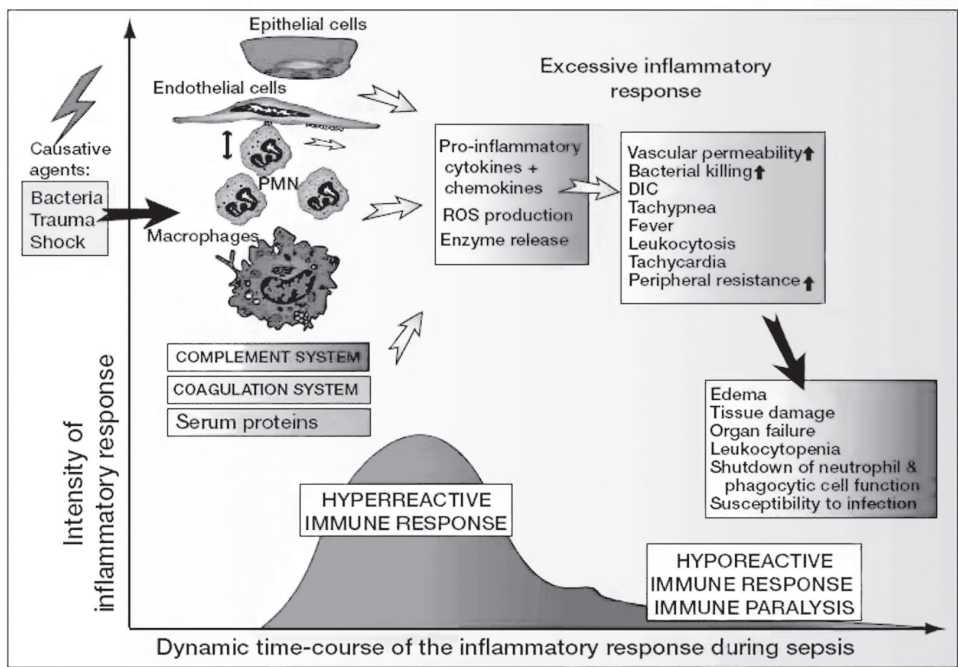


Figure 2. Dynamic time-course of the inflammatory response during sepsis. Various stimuli can cause activation of different cell types and serum proteins, as well as of the coagulation and

complement system, leading to excessive production of pro-inflammatory cytokines and chemokines and up-regulation of adhesion molecules on endothelial cells and polymorphonuclear leukocytes (PMNs). Monocytes and PMNs release large amounts of granular enzymes and generate ROS in response to the original stimulus in the early (hyperreactive) phase of sepsis. As result of excessive pro-inflammatory mediator production, vascular permeability increases, tissue damage and organ failure occur and crucial innate immune functions become defective, resulting in increased susceptibility toward infection in the later (hyporeactive) phase of the immune response, often along with the occurrence of immune paralysis. DIC, disseminated intravascular coagulopathy. *Reprinted with permission from Riedemann NC, Guo RF, Ward PA.(2003) Novel strategies for the treatment of sepsis. Nature Medicine;9:517-24. © 2003 Nature Publishing Group.*

Dysregulated Coagulation

In normal hemostasis there is a balance between the blood being fluid to allow free flow within the vessels and appropriate clotting to control bleeding (for more details on molecules involved in coagulation, see chapter 1.1.4). Inflammation can cause significant alterations at multiple levels within the coagulation system and the cells that regulate this system ⁴⁸. Septic patients frequently manifest disseminated intravascular coagulation (DIC) with consumption of platelets and prolongation of clotting times. In addition, the altered hemostasis leads to an inappropriate blood clotting, which results in clogged blood vessels and reduced blood flow. The interaction between the clotting system, circulating white blood cells and platelets, and the endothelium, covering all blood vessel walls in our body, adds another level to an already multifaceted picture. Due to the severe coagulation abnormalities, clinical trials with anticoagulant recombinant activated protein C (APC), which inactivates factors V and VIII of the clotting cascade, have been initiated following studies in the baboon model of *Escherichia coli* sepsis ^{49, 50}. APC turned out to improve survival in patients with severe sepsis, but it is clearly not a panacea for all patients ⁵¹. The most beneficial effects were observed in patients with the worst prognosis. On the other hand, patients at low risk for death had no improvement in survival and a significantly increased risk for bleeding when treated with APC ⁵¹.

Cellular Dysfunction

Many cellular aspects become dysfunctional in sepsis. The induction of cellular apoptosis and necrosis is one of the areas of active investigation and has been reviewed elsewhere in detail ⁵². Increased apoptosis may contribute to the pathogenesis of sepsis by delayed removal of those cells that should be removed, e.g., neutrophils, and early removal of those cells that should not be removed, e.g., lymphocytes.

Significant apoptosis of lymphocytes has been demonstrated in septic patients. These apoptotic lymphocytes were observed in all lymphoid organs including the spleen and thymus, but also in the gastric associated lymphatic tissue ⁴¹. Blocking lymphocyte

apoptosis in peritonitis improved survival in murine sepsis models^{53,54}. Consequently, it has been speculated that prevention of apoptosis may be efficacious in sepsis by preventing immune suppression that occurs in the later phase of the immune response in sepsis⁵⁵.

Polymorphonuclear neutrophils (PMN) with their potent oxidative and proteolytic potential are a critical component of the innate immune response to infectious challenges. Neutropenic patients, regardless of the cause of the neutropenia, and patients with PMN dysfunction are at increased risk for the development of infectious complications⁵⁶. An appropriate, strong PMN activation benefits the patient and helps to eradicate an infectious focus. It is difficult to define an appropriate response versus a hyperactive response⁵⁷. Patients who have suffered traumatic injury are at increased risk for the development of multisystem organ failure due to increased chemotactic responses of PMN to CXC chemokines⁵⁸. However, PMN isolated from septic patients demonstrate decreased chemotaxis toward IL-8 and depressed expression of CXCR2⁵⁹. In accordance with the above, it was confirmed in early trauma patients that high CXCR2 function correlated with the development of organ injury, e.g. acute respiratory distress syndrome, whereas low function predisposed to pneumonia and sepsis⁶⁰.

Another significant matter concerns PMN survival since inappropriate apoptosis of PMN occurs in septic patients. Neutrophils in the circulation typically have a short lifespan of only 5–6 hours following their maturation and release from bone marrow stores, and ≤24–36 hours when cultured *in vitro*⁶¹. However, patients with sepsis have a delay in their neutrophil apoptosis, causing them to persist longer in the bloodstream. One of the reasons is a prolonged activation of nuclear factor κ B (NF κ B) and reduced caspase-3 activity⁶². Consequently, the prolonged exposure of organs to the cytotoxic factors produced by PMN can lead to organ injury. So, on one side, the oxidant potential of neutrophils is increased, while at the same time functions such as chemotaxis and phagocytosis are often depressed^{63,64}.

1.1.3 SEPSIS AND MICROCIRCULATORY DYSFUNCTION

The microcirculation constitutes a functionally highly active system that dynamically interacts with circulating and tissue-associated cells (i.e., leukocytes, platelets, endothelial cells), and contributes to local, downstream and even upstream regulation of vascular tone. When this system is damaged, it can affect all participating cellular components, in particular endothelial cells, smooth muscle cells, as well as circulating blood cells. Activation, dysfunction and injury of microvascular endothelial cells may occur as a result of ischemia, inflammatory mediators, as well as adherent leukocytes, in particular neutrophils with their attribute to produce cytotoxic factors and proteolytic enzymes as mentioned in 1.1.2. Consequences of microcirculatory dysfunction include

the breakdown of endothelial and epithelial barrier function, leading to tissue edema and uncontrolled inflammatory cell infiltration. Furthermore, vasodysregulation occurs leading to the formation of arteriovenous shunts and/or the loss of peripheral resistance with severe macrohemodynamic consequences, disturbance of oxygen transport and utilization by tissue cells.

In sepsis circulatory disturbances, including decreased peripheral vascular resistance and maldistribution of blood flow, as well as disturbance of oxygen transport occur, leading to focal tissue hypoxia and cell injury. This dysfunction of microcirculation is one feature of the sepsis-related multiorgan failure ⁶⁵. The characteristic macrohemodynamic feature of sepsis in its advanced manifestation is a vasodilatory shock, characterized by hypotension as a result of loss of peripheral resistance due to the failure of vascular smooth muscle to constrict ^{66, 67}. The loss of peripheral resistance occurs despite markedly increased blood levels of catecholamines ⁶⁸ and is characterized by a poor response of the vascular smooth muscle cells to exogenous administration of catecholamines and other vasopressor agents ^{69, 70}. The loss of peripheral resistance can be linked to a maldistribution of blood flow at the microcirculatory level. In order to pass through the smallest segment of the microcirculation, i.e., the capillaries, blood cells are required to undergo changes in shape and circumference. The deformability of red blood cells (RBC) is clearly reduced in septic adult patients ^{71, 72} as well as in septic infants ⁷³. The mechanisms involved in this RBC rigidity in sepsis have not been thoroughly delineated and may include peroxidation of RBC membrane lipids, increased cytosolic calcium concentrations as well as NO overproduction ⁷⁴⁻⁷⁷. Also, activated neutrophils, which can be found in increased numbers in the peripheral blood of septic patients show reduced deformability, thus contributing to microcirculatory dysfunction in sepsis ⁷³. Likewise, leukocyte activation results in increased aggregability, which may contribute to vascular obstruction and impaired microvascular flow, and an increased expression of adhesion molecules on the surface of neutrophils of septic patients ⁷¹. Finally, septic patients often show DIC, which contributes to microcirculatory dysfunction through fibrin deposition and the occlusion of capillaries by microthrombi ^{71, 78}. Histological and ultrastructural studies on muscle and skin biopsies of septic patients demonstrated that myocyte and capillary damage is associated with the breakdown of endothelial barrier function and the local accumulation of neutrophils, macrophages, and mast cells ⁷⁸. This loss of endothelial barrier function and increased microcirculatory permeability has also been confirmed in a study on septic patients using computer-assisted venous congestion plethysmography ⁶⁵. Since microcirculatory obstruction develops at the level of capillaries, blood is thought to bypass the microcirculation through large emerging arteriovenous shunts, thereby contributing little to tissue oxygenation.

In summary, the microcirculatory dysfunction in sepsis is characterized by loss of peripheral vascular resistance, maldistribution of blood flow, reduced deformability of

RBC and PMN, DIC, increased microcirculatory permeability and microthrombus formation.

1.1.4 SEPSIS AND ENDOTHELIAL DYSFUNCTION

The endothelium covers the surface of all blood vessels in our body and therefore resides at the critical interface between blood and the tissue ⁷⁹. Due to this location endothelial cells have an intense contact with blood cells and plasma proteins. Under physiologic conditions, endothelial cells exert various functions that are important for normal homeostasis. These functions include the maintenance of blood fluidity by prevention of coagulation, the orchestration of the migration of blood cells into the tissues by expression of adhesion molecules, the regulation of the microcirculation by controlling the tonus of the arterioles, and the regulation of vasopermeability ⁸⁰.

Under physiologic conditions, endothelial cells maintain blood fluidity by various mechanisms that inhibit coagulation throughout the vascular system (fig 3.). Endothelial cells express tissue factor pathway inhibitors (TFPIs) that block the initiation of coagulation (Fig. 3a). They have proteoglycans, such as heparan sulfate, on their surface, which bind anti-thrombin III and inactivate thrombin ⁸¹ (Fig. 3a). They express thrombomodulin, a membrane protein, which binds thrombin and modifies its specificity as a procoagulant converter of fibrinogen to fibrin into an anticoagulant activator of protein C ⁸² (fig. 3a). Protein C inactivates in the presence of its cofactor protein S activated factors V and VIII of the clotting cascade ⁸³. The endothelium also inhibits platelet adhesion and aggregation by producing nitric oxide (NO), generated by nitric oxide synthase 3 (NOS3)-mediated conversion of arginine, and prostacyclin ⁸⁴. This process is also supported by the expression of a surface-bound adenosine diphosphatase, which hydrolyzes an important agonist of platelets, adenosine diphosphate ⁸⁵ (fig. 3a). NO also serves as a vasodilating agent as well as prostacyclin, which is also produced by endothelial cells. By these two vasodilating agents, the endothelium regulates the tonus of the arterioles via the smooth muscle cells in the medial layer of the vessel wall (fig. 3b), thereby regulating the microcirculation and decreasing blood pressure ⁸⁶. Furthermore, a capillary endothelial barrier, which is formed in most tissues by intercellular junctions containing tight and adherens junctions, prevents the passage of plasma proteins to tissues (fig 3c). Resting endothelial cells do not interact with leukocytes ⁸⁷, because they sequester leukocyte-interactive proteins, such as P-selectin and von Willebrand factor (vWF) within Weibel-Palade bodies (WPBs) (fig. 3d). The transcription of other adhesion molecules, such as E-selectin, vascular cell-adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 is also suppressed by resting endothelial cells.

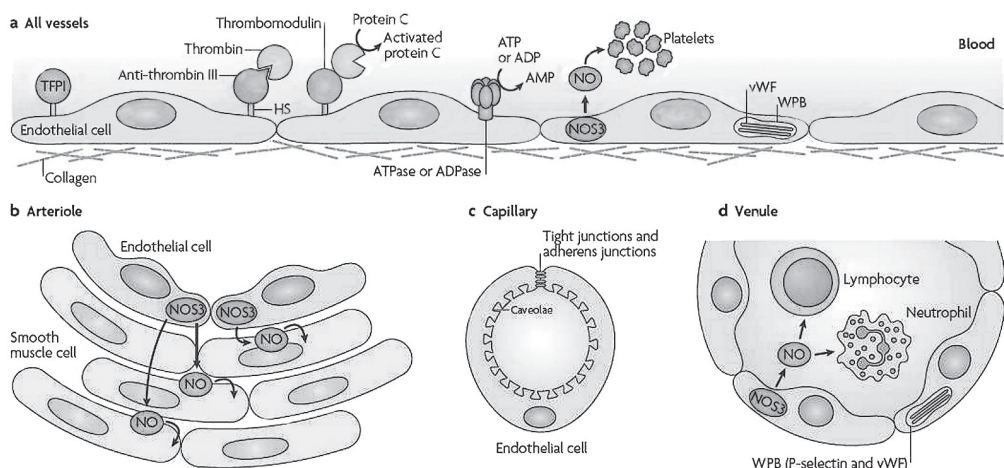


Figure 3. Functions of resting endothelial cells. (a) All endothelial cells (EC) inhibit coagulation of the blood by expressing tissue factor pathway inhibitors (TFPIs), displaying heparan sulphate proteoglycans (HS) and Thrombomodulin on their surface, producing nitric oxide-synthase 3 (NOS3)-mediated nitric oxide (NO) and sequestering von Willebrand factor (vWF) within their storage granules, known as Weibel–Palade bodies (WPB). (b) Arterial endothelial cells have a major role in regulating blood flow by controlling the tone of smooth muscle cells in the medial layer of the vessel wall by NOS3 produced NO. (c) Capillary EC are the principal regulators of transendothelial extravasation of plasma proteins by forming intercellular junctions. (d) Venular EC form the principal site of leukocyte trafficking from the blood into the tissues. Efficient recruitment of leukocytes requires that the EC are activated and express adhesion molecules that mediate leukocyte attachment. Resting EC sequester P-selectin into Weibel–Palade bodies (WPB) along with vWF. See text for details. *Reprinted with permission from Pober JS and Sessa WC. (2007) Evolving functions of endothelial cells in inflammation. Nature Review Immunology;7:803-15. © 2007 Nature Publishing Group.*

Sepsis and systemic inflammation induce rapid and profound changes in endothelial function. Upon interaction with inflammatory mediators, such as $\text{TNF-}\alpha$ or IL-1 , the endothelium will become activated. By the loss of thrombomodulin^{88, 89} and heparan sulfate⁹⁰ it loses its anticoagulant properties and becomes a procoagulant surface. This is further intensified by the synthesis of tissue factor (TF) on the surface^{91, 92}. Consequently, endothelial cells lose the clotting inhibitors TFPIs and antithrombin III, and the ability to activate protein C, and by the interaction of TF with clotting factor VII/VIIa they activate the extrinsic pathway of the coagulation system. Furthermore, the production of the vasoactive compounds that regulate the tonus of the arterioles,

including the vasodilators NO and prostacyclin, and the vasoconstrictor endothelin is strongly modified ⁹³. Under normal conditions, endothelial cells produce NO by a constitutive NO-synthase, eNOS, which is calcium-dependent ⁹⁴. Upon stimulation with pro-inflammatory cytokines endothelial cells may produce the inducible NO synthase (iNOS), which produces large amount of NO in a calcium-independent manner ⁹⁵ leading to rapid hypotension ⁹⁶. Also, increased levels of the vasoconstricting endothelins have been described in septic patients ⁹⁷, which may compromise the appropriate matching of flow to tissue needs ⁹⁸. Another very important alteration upon cytokine stimulation is the expression of adhesion molecules, such as P-Selectin, E-Selectin, VCAM-1 and ICAM-1 on the membrane of endothelial cells ⁹⁹. This leads to leukocyte binding, rolling over the endothelium, followed by strong adherence and finally the transmigration into the tissues ¹⁰⁰ enabling the leukocyte to enter the site of infected or damaged tissue (fig. 4). The activated endothelium further produces an array of inflammatory mediators, including cytokines, chemokines and complements factors, which all take part in the inflammatory response. As the level of inflammatory mediators produced by endothelial cells is highly heterogenous ¹⁰¹, severity of inflammation in sepsis could be dependent on interindividual variations of endothelial cells to respond to inflammatory mediators.

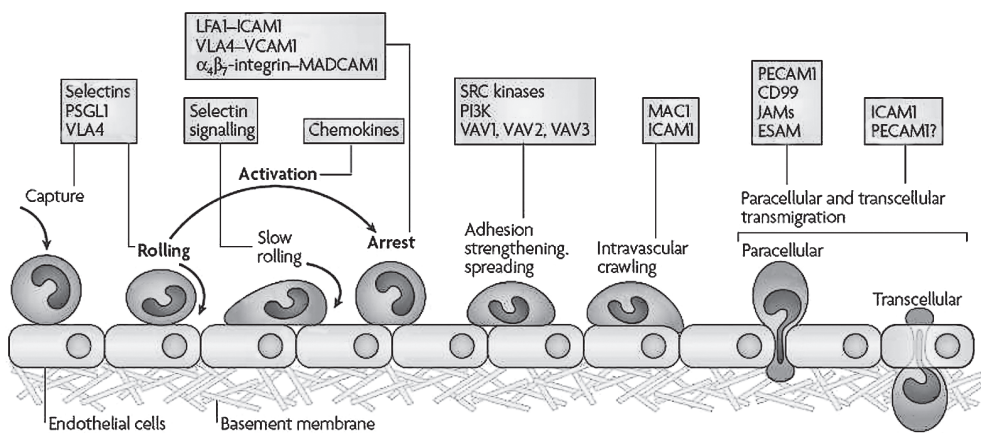


Figure 4. The leukocyte adhesion cascade. Upon stimulation with inflammatory mediators, adhesion molecules on endothelial cells will be up-regulated leading to interaction with leukocytes. This induces a cascade of different steps resulting in the transmigration of leukocytes into the tissue. The key molecules involved in each step are indicated in boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as $\alpha L\beta 2$ -integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular

addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as $\alpha 4\beta 1$ -integrin). Reprinted with permission from Ley K, Laudanna C, Cybulsky MI, Nourshargh S. (2007) *Getting to the site of inflammation: the leukocyte adhesion cascade updated*. *Nature Review Immunology*;7:678-89. © 2007 Nature Publishing Group.

Endothelial activation is a physiological process in response to inflammatory mediators resulting in recruitment of leukocytes to the site of infection to eradicate the microbes. Several mechanisms may lead to endothelial dysfunction or damage, at which the permeability may become impaired leading to capillary leakage which complicates sepsis.

In vitro, endothelial cells will undergo apoptosis in response to several mediators, including some infectious agents. First of all, neutrophils, which adhere to endothelial cells via adhesion molecules are well able to cause endothelial damage by producing oxygen radicals and proteinases such as elastase, thereby inducing apoptosis¹⁰². These degranulation products of neutrophils were increased in septic patients and correlated with clinical outcome¹⁰³. Also, pro-inflammatory cytokines such as TNF- α can induce apoptosis of endothelial cells *in vitro*¹⁰⁴. Furthermore, endothelial apoptosis can be induced by ischemia/reperfusion injury, since reperfusion of ischemic tissues can increase the local inflammatory reactions causing additional damage¹⁰⁵.

The morphological damage pattern includes the deformation and loosening of the endothelial cell texture, apoptosis and consequent destruction of the cell structure, and phagocytosis¹⁰⁶. In addition, destruction and dearrangement of intercellular contact molecules, and proteolysis of endothelial basal membrane can lead to detachment of endothelial cells^{107, 108}. In fig. 5 mouse aortic endothelium is shown under septic conditions induced by cecal ligation and puncture (CLP) compared to healthy endothelium. The CLP technique is a common method to induce endotoxic shock and represents a model of sepsis which closely reproduces the clinical situation¹⁰⁹. By ligation and perforation of the cecum, feces will be allowed to enter the abdominal cavity and induce a pro-inflammatory response leading to endotoxic shock. The electron microscopic analysis shows significant morphologic abnormalities in the structure of the aortic endothelium after CLP including destruction of the endothelial cells and their detachment from the basal membrane.

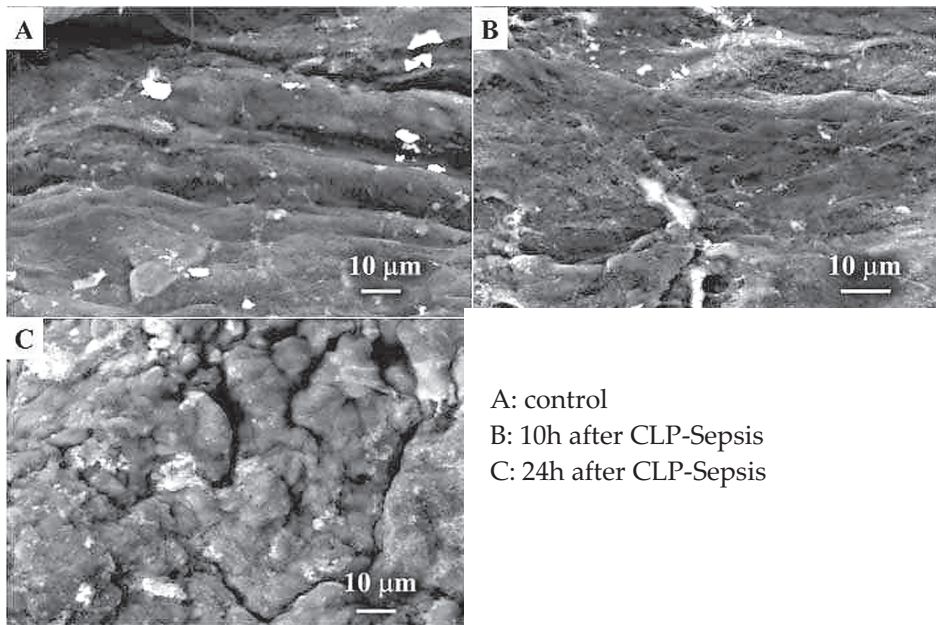


Figure 5. Electron microscopic analysis of normal mouse aortic endothelium and aortic endothelium of mice subjected to septic shock. Mice were subjected to sham operation or cecal ligation and puncture (CLP). Aortic endothelium is shown of control mice (A), and mice 10 h (B) and 24 h (C) after subjected to CLP. The electron microscopic analysis shows partial detachment of some endothelial cells from the basal membrane at 10 h after CLP (B). The structure of the aortic endothelium 24 h after CLP exhibits a more significant morphologic abnormality (C), with most endothelial cells being swollen and appearing to be partially detached from the basal membrane. Reprinted with permission from Matsuda N, Hattori Y. (2007) *Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction*. *J Smooth Muscle Res*;43:117-37 and by courtesy of Prof. Yuichi Hattori, Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences University of Toyama, Japan.

In the process of destruction and detachment of endothelial cells, apoptosis and necrosis eventually lead to release of endothelial cells or endothelial microparticles into the circulation. This, in turn, can induce an inflammatory tissue reaction due to increased expression of adhesion molecules on the microparticles leading to enhanced interactions between leukocytes, endothelial cells and their microparticles. This apoptosis- and necrosis-induced endothelial cell damage could be confirmed clinically in septic patients, in which increased levels of vWF- and vascular endothelial growth factor receptor (VEGFR)-2-positive circulating endothelial cells could be identified during sepsis and septic shock¹¹⁰. Also, the enhanced production of endothelial microparticles

with increased binding to leukocytes could be confirmed in patients with severe systemic inflammatory response syndrome (SIRS) ¹¹¹.

Endothelial dysfunction and detachment of endothelial cells result in the development of interstitial edema. The decreased production of important anti-coagulative proteins and the dysfunction of fibrinolysis supports the coagulation-activating effects of cytokines. Eventually, these processes induce severe pathological alterations in the microcirculation with dysfunctions in oxygen-transport culminating in hypoxic organ-failure ¹¹². Although these alterations seem to be similar in all septic patients, an improvement of the capillary perfusion was only found in sepsis-survivors, but not in sepsis-non-survivors ¹¹³. This finding supports the assumption, that endothelial dysfunction leading to impaired capillary perfusion plays an important role in the development of multi-organ-failure in the course of sepsis ¹¹⁴.

1.1.5 THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN SEPSIS

As mentioned in the chapter 1.1.4, altered endothelial function appears in the macro- and microcirculation in the course of sepsis and includes detachment of endothelial cells and capillary leakage. In these pathologic conditions, reconstitution of the endothelial layer is initiated. The recruitment of endothelial progenitor cells (EPCs) might play a role in this process, since many *in vivo* studies have demonstrated that vascular maintenance, repair, and neovascularization in ischemic tissue are partly mediated by recruitment of EPCs ¹¹⁵⁻¹¹⁷.

Ever since the description of postnatal vasculogenesis, a process in which EPCs are recruited and differentiate into mature endothelial cells (EC) to form new blood vessels ^{115, 117, 118}, growing evidence suggests the involvement of EPCs also in other pathological conditions. EPCs may also be recruited and incorporated into sites of active neovascularization during e.g., vascular trauma, tumor growth and inflammation. Moreover, expansion and mobilization of EPCs might represent an effective method to stimulate angiogenic activity of resting mature endothelial cells ^{119, 120}.

The majority of EPCs reside in the bone marrow in close association with hematopoietic stem cells (HSC) and the bone marrow stroma. EPCs have the capacity to proliferate, migrate and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature endothelial cells (EC), such as VE-cadherin or vWF. In animal ischemia models, mobilization of bone marrow-derived EPCs resulted in EPC homing to sites of active neovascularization and differentiation into EC ¹²¹. Mobilization of EPCs from the bone marrow critically depends on the activation of metalloproteinases and up-regulation of adhesion molecules. This is most likely mediated by soluble factors such as VEGF, granulocyte-macrophage colony stimulating factor (GM-CSF) and

erythropoietin (EPO). Serum concentrations of these factors dramatically increase under pathologic conditions, concomitantly with an increase in the number of cEPCs¹²²⁻¹²⁴. Many other factors are described to have important roles for the mobilization of EPCs. Among them are placental growth factor¹²⁵, angiopoietin-1¹²⁶, pro-inflammatory cytokines such as G-CSF¹²⁴, chemokines such as stromal cell-derived factor (SDF)-1¹²⁷, and hormones such as estrogens¹²⁸. Interestingly, lipid-lowering¹²⁹ and anti-diabetic drugs¹³⁰, as well as physical activity¹³¹ also stimulate EPC mobilization.

Definition and characterization of endothelial progenitor cells

EPCs represent with 0.001-0.05% a minor subpopulation of blood mononuclear cells (MNCs)¹³². *In vitro* culture methods have been developed to select and expand this population. Three culture methods have been described for isolating EPCs (fig. 6)¹³³. The original method by Asahara et al.¹¹⁵ has been modified subsequently by others^{134, 135} and the resulting colonies, comprised of round cells centrally with spindle-shaped cells sprouting at the periphery, are nowadays referred to as colony-forming unit-ECs (CFU-ECs) or colony-forming unit-Hill cells¹³⁴. These CFU-EC colonies could be cultured from a heterogeneous MNC population enriched for either CD34 or VEGFR-2. Another widely employed and methodologically similar approach, has been used in several studies^{117, 136, 137}. Unfractionated MNCs are cultured in supplemented endothelial growth media for 4 days, whereupon the non-adherent cell fraction is removed. The resulting culture of adherent cells display features of an endothelial phenotype through binding of the endothelial-specific lectin Ulex Europaeus Agglutini-1, and uptake of acetylated low-density lipoprotein (acLDL)^{117, 137}. These cells appear similar to CFU-ECs in surface marker expression and in *in vitro* function, and as a result, both have often been grouped together in the literature under the name EPCs. These adherent cultured cells have also been referred to as circulating angiogenic cells (CAC) in recognition that these cells appear to promote neovascularization in animal models of critical limb ischemia or myocardial infarction (MI)¹¹⁷. A third type of EPCs is termed 'endothelial colony-forming cells' (ECFCs). To obtain these, MNCs are plated onto collagen I-coated plates in endothelial-specific growth media. Non-adherent cells are discarded during washing steps. ECFC colonies emerge from the adherent cell population 10-21 days after plating and display cobblestone appearance typical for ECs^{138, 139}. Thus far, ECFCs have proven to be phenotypically indistinguishable from mature ECs, and exhibit *de novo* vessel-forming ability^{140, 141}. Because ECFCs emerge much later in culture as compared to both CFU-ECs and CACs, ECFCs have been called 'late outgrowth' EPCs, while CFU-ECs and CACs have been called 'early outgrowth' EPCs¹⁴²⁻¹⁴⁴.

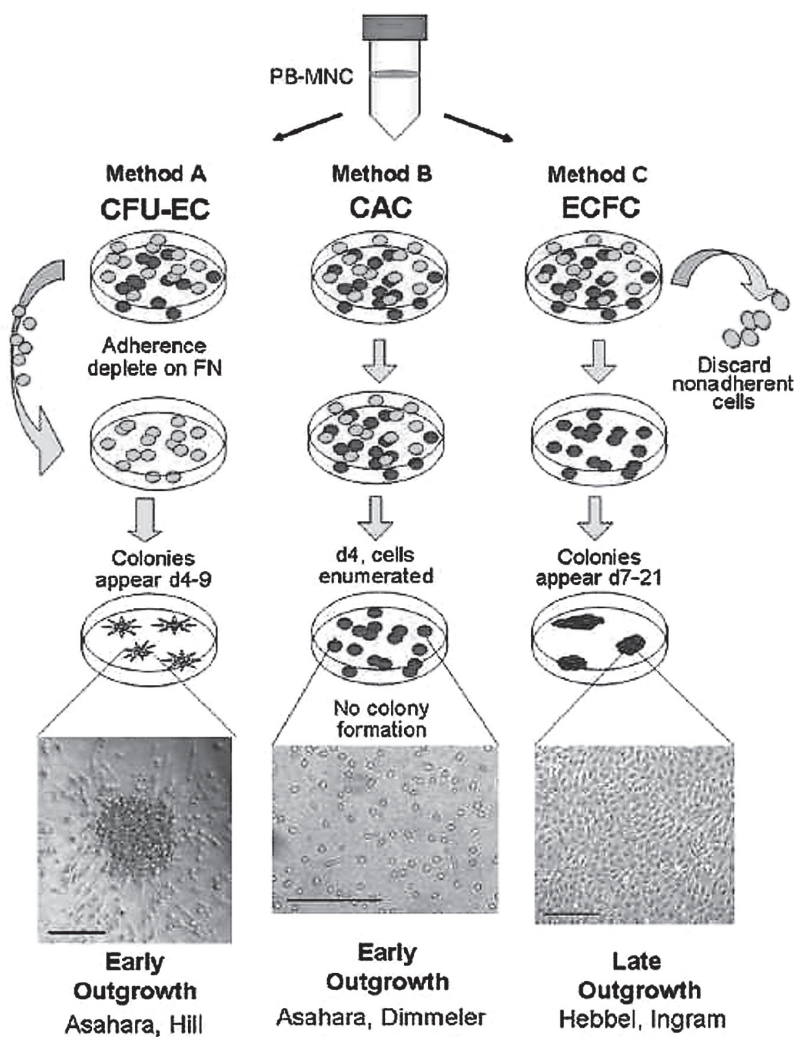


Figure 6. *In vitro* methods of EPC culture. (Method A) Culture of colony-forming unit – endothelial cells (CFU-EC) includes a 5-day process wherein non-adherent MNCs give rise to the EPC colony. (Method B) Circulating angiogenic cells (CAC) are the adherent mononuclear cells of a 4- to 7-day culture procedure. CAC cultures typically do not display colony formation. (Method C) Endothelial colony-forming cells (ECFCs) are derived from adherent MNCs cultured for 7–21 days in endothelial conditions and colonies display a cobblestone morphology. Reprinted with permission from Prater DN, Case J, Ingram DA and Yoder MC. (2007) Working hypothesis to redefine endothelial progenitor. *Leukemia*;21:1141-1149. © 2007 Nature Publishing Group.

When using cell culture to define and characterize EPCs, the starting cell population is typically heterogeneous, making it difficult to determine the precursor cell that gives rise to the final EPCs. Alternatively, EPCs can be prospectively identified without the need for culture, by selecting sub-populations of MNCs based on specific cell surface antigen expression. The definition and characterization of EPCs by utilizing cell surface antigens as markers allows for the selection of a more homogenous population. However, the definition of EPCs via this method is complex due to the absence of any single and restricted surface marker for EPCs versus detached circulating ECs (CECs). Furthermore, it is complicated due to the overlap observed between cell surface antigens expressed on the surface of putative EPCs and those expressed on cells of the hematopoietic lineage¹⁴⁵⁻¹⁴⁷. While the cell surface antigens CD34, CD133, and VEGFR-2 are utilized to identify EPCs, it is important to note that they are also expressed on human hematopoietic stem cells (HSCs), as well as on various sub-populations of hematopoietic progenitor cells (HPCs), including low proliferative potential- and high proliferative potential-colony forming cells¹⁴⁶⁻¹⁴⁸.

Human circulating EPCs have typically been identified as cells expressing CD34, CD133, and VEGFR-2¹⁴⁸⁻¹⁵¹ (fig. 7). Peichev et al.¹⁴⁹ reported that mature ECs do not express CD133, and that nearly all the CD34+/VEGFR-2+ circulating EPCs in peripheral and human umbilical cord blood express CD133. Furthermore, culture of CD34+ cells from human fetal liver with VEGF and fibroblast growth factor-2 (FGF-2) resulted in differentiation of non-adherent CD133+/CD34+ cells into adherent CD133-/VEGFR-2+/acetylated LDL-uptake+ cells with an EC morphology¹⁴⁹. In an *in vivo* human model, the neo-intima formed on the surface of left ventricular assist devices was found to be colonized with CD133+/VEGFR-2+ cells¹⁴⁹. These data suggested that the circulating CD34+ cells co-expressing CD133 and VEGFR-2 are a phenotypically and functionally distinct population of EPCs that may play a role in neovascularization.

Many clinical studies designed to determine the role of EPCs in a variety of vascular disorders have used flow cytometry to investigate and enumerate the EPC concentration in the blood of patients with vascular disease¹⁵¹⁻¹⁵⁴. In these studies, the widely accepted definition of EPCs co-expressing CD34, CD133, and VEGFR-2 has been applied. But since these antigens are also expressed on primitive hematopoietic progenitor cells (HPCs), Case et al.¹⁵⁵ isolated CD34+/CD133+/VEGFR-2+ cells from human umbilical cord blood and granulocyte colony-stimulating factor-mobilized peripheral blood, and assayed them for either EPCs or HPCs. They found that CD34+/CD133+/VEGFR-2+ cells did not give rise to early outgrowth EPCs and were devoid of vessel forming activity. In contrast, CD34+/CD133+/VEGFR-2+ cells gave rise to HPCs that expressed the hematopoietic lineage-specific antigen CD45. Therefore, they tested whether EPCs could be separated from HPCs by immunoselection for CD34 and CD45. Their results showed that CD34+/CD45+ cells gave rise to HPCs but not EPCs, while CD34+/CD45- cells gave rise to EPCs but not HPCs. These data, in contrast to the observations of Peichev et al.¹⁴⁹,

lead to the conclusion that CD34+/CD133+/VEGFR-2+ cells represent HPCs that do not yield EC progeny.

In summary, flow cytometry and colony forming assays are the two main methods used for identification and functional assessment of endothelial progenitor cells. However, both of these techniques have serious limitations. Although endothelial cells-colony forming units (EC-CFU) are widely accepted as a surrogate estimate of EPC number and function in cell culture, some important limitations may restrict the assumption that EC-CFUs reflect EPC numbers accurately. Shantsila et al ¹⁵⁶, comparing CFU units to flow cytometry, described that endothelial CFU counts represent the cumulative characteristics of EPC quantity and their functional characteristics, and cannot be reliably used for the estimation of EPC numbers in peripheral blood or the bone marrow. They suggest that flow cytometry may be the better technique for EPC quantification.

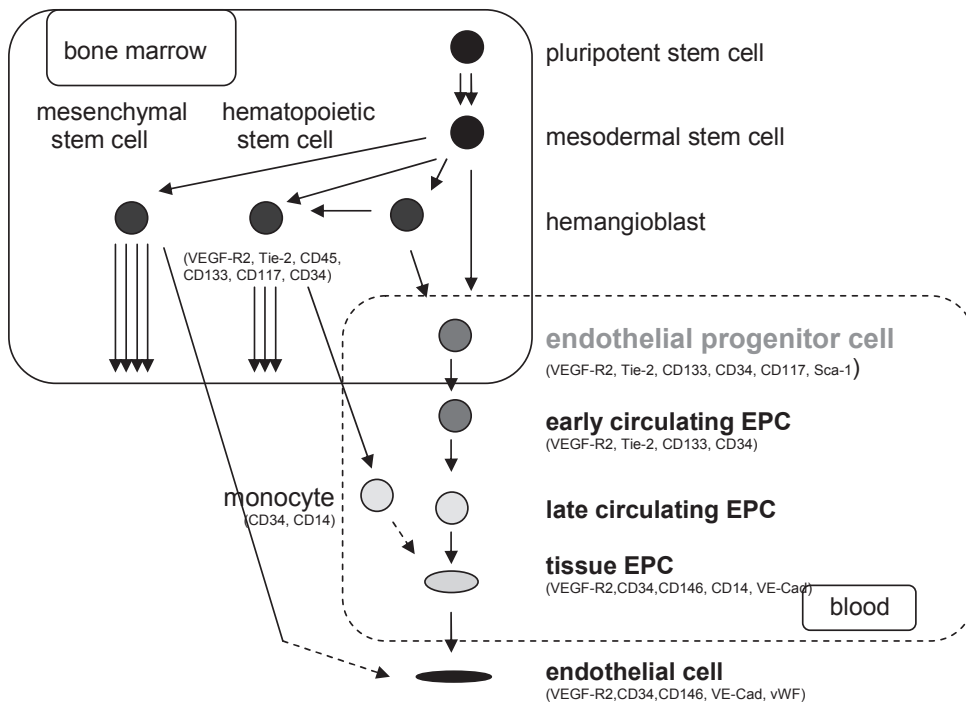


Figure 7. Characterization and differentiation of endothelial progenitor cells. Reprinted with permission from Beck GC, Rafat N, Yard B, Hanusch C. (2007) The role of endothelial progenitor cells in sepsis. *Anaesthesist*;56:423–428. © 2007 Springer Medizin Verlag; adapted with permission from Asahara T, Kawamoto A. (2004) Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol*;287:C572-579. © 2004 The American Physiological Society.

Homing of endothelial progenitor cells to activated endothelium

Homing of endothelial progenitor cells to the target tissue is a multi-step-process involving various chemokines, cytokines, adhesion molecules and proteases (fig. 8). While the homing of leukocytes to sites of inflammation is well studied (see chapter 1.1.4, fig. 4), the molecular mechanisms of progenitor cell homing to sites of ischemia or injury are poorly understood. From a process point of view the homing of endothelial progenitor cells to sites of ischemia and to sites of injury share at least some common features with the homing of leukocytes to sites of inflammation. In a mouse model, embryonic EPCs (eEPC) arrested within tumor microvessels extravasated into the interstitium and incorporated into neovessels suggesting that adhesion and transendothelial migration are also involved in the recruitment of endothelial progenitor cells to sites of tumor angiogenesis¹⁵⁷. The fact that these cells can contribute to tumor angiogenesis indicates that eEPCs, although they are primarily programmed to form blood vessels during embryonic vascular development, retain this ability within an angiogenic environment in the adult and may be comparable to adult EPCs¹⁵⁷.

Also, adhesion molecules, which play a critical role in rolling a firm adhesion and transmigration of leucocytes were identified as key regulators of EPC homing. Recently, evidence was provided that the initial steps of this process are mediated by P-selectin and E-selectin¹⁵⁸. Activation of the ephrin family member EphB4 in EPCs leads to a higher expression of PGSL-1, a selectin ligand, leading to an increased adhesion to P-selectin and E-selectin. siRNA for P-selectin suppresses this response indicating that PGSL-1 expression facilitates the recruitment of EPCs and thus enhances their proangiogenic capacity¹⁵⁸. Other studies have shown that also E-selectin potentiates angiogenesis in ischemic hindlimbs, partly by mediating EPC-endothelial cell interactions¹⁵⁹.

β 2-integrins expressed on the cell surface of EPCs mediate the firm adhesion and transmigration of EPCs to the damaged endothelial monolayer. Activation of the β 2 integrins was shown to improve the homing and the neovascularization capacity of EPCs in a mouse model of hindlimb ischemia¹⁶⁰. Interestingly, HMGB-1, which is released extracellularly upon activation of cells by inflammatory cytokines and during cell necrosis, and a late marker of sepsis, was recently reported to activate β 1 and β 2 integrins on the surface of endothelial progenitors, thereby guiding EPC adhesion and homing to ischemic areas¹⁶¹. The importance of β 2 integrins in homing of EPCs is also highlighted by studies focusing on ICAM-1. Up-regulation of ICAM-1 during ischemia was shown to associate with enhanced EPC recruitment to ischemic limbs¹⁶².

α 4 integrin, expressed on EPCs, also seems to play a crucial role in progenitor cell homing. It promotes homing of circulating endothelial progenitors to the sites of active tissue remodelling¹⁶³ and improves blood flow recovery and tissue preservation¹⁶⁴. This supports the assumption, that interaction between EPC surface molecules with their

counter ligands on the dying endothelial cells or sub-endothelial matrix proteins plays a major role in EPC homing.

The receptor c-Kit and its membrane-bound form of Kit ligand (KitL) are involved in the mobilization of EPCs from the bone marrow ¹⁶⁵. Observations that the soluble KitL also promotes endothelial cell migration and survival ¹⁶⁶ raised the possibility that c-Kit and KitL might be also involved in EPC homing to activated endothelium. Recently, Dentelli et al. ¹⁶⁷ demonstrated that inflammatory activation induced the expression of the KitL on microvascular endothelial cells in vitro and in vivo. The inflammatory activation mediated also the recruitment of EPCs. Moreover, they showed that depletion of endogenous c-Kit or inhibition of c-Kit enzymatic activity prevented adhesion of EPCs to activated ECs both in vitro and in vivo, indicating that functional c-Kit on EPCs is essential. Since the chemokine SDF-1 mediates homing of stem cells to bone marrow by binding to CXCR4 on circulating cells ¹⁶⁸, Ceradini et al. ¹²⁷ investigated the regulation of SDF-1 expression and its physiological role in peripheral tissue repair. They showed that the recruitment of CXCR4-positive EPCs to regenerating tissue is mediated by hypoxic gradients via HIF-1-induced expression of SDF-1.

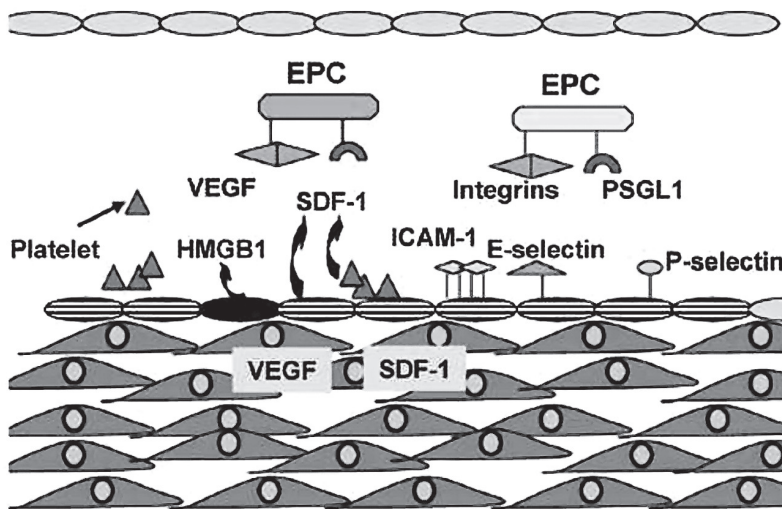


Figure 8. EPC homing to activated endothelium. After vascular injury causing local inflammation, the endothelial monolayer is activated as a consequence of which rapid platelet aggregation occurs. Platelets and activated endothelial cells secrete high levels of SDF-1, while elevated levels of VEGF are also observed. PGSL-1, a ligand for selectins, is up-regulated in these cells through activation of the EphB4 pathway. Extremely high levels of $\beta 2$ integrins can also be found in EPCs. All these molecules will interact with their ligands P-selectin, E-selectin, and ICAM-1 that are expressed on the activated endothelial cells. Additionally, necrotic ECs express high mobility group box protein (HMGB)-1 that further enhances the interaction of $\beta 2$

and $\beta 1$ integrins with their ligand ICAM-1. Reprinted with permission from Zampetaki A, Kirton JP, Xu Q. (2008) Vascular repair by endothelial progenitor cells. *Cardiovasc Res.*;78:413-421. and by courtesy of Prof. Qingbo Xu, Cardiovascular Division, Kings College London, UK. © 2008 The Author.

EPCs need to transmigrate to the injured tissue to be able to influence neovascularization. Their invasive capacity is crucial for tissue repair and restoration of organ function. Cathepsin L was reported to play an important role in this process. This protease is highly expressed in EPCs and essential for matrix degradation and invasion. Cathepsin L-deficient mice displayed impaired recovery following hindlimb ischemia, and infused cathepsin L-deficient EPCs neither homed to sites of ischemia nor augmented neovascularization ¹⁶⁹.

MMP-2 was also found to affect the invasive properties of endothelial progenitors ¹⁷⁰. EPCs from MMP2-/- mice exhibit reduced extracellular matrix degradation and as a result, MMP2-/- mice respond poorly to hindlimb ischemia because of reduced neoangiogenesis ¹⁷⁰.

In conclusion, homing of EPCs is a complex process and involves various mediators that recruit them to activated endothelium in response to a damage-induced inflammation. EPCs have been extensively studied in cardiovascular diseases and accumulating evidence highlights their importance in vascular repair and tissue remodeling.

The role of EPCs in sepsis has not been studied so far. In patients with acute lung injury (ALI) EPC colony numbers were significantly higher compared with healthy control subjects, but did not differ between patients with ALI and intubated control subjects. Increased cEPC numbers were associated with improved survival in the ALI group. Septic shock was present in 44% of the patients with ALI and its incidence was statistically not different in survivors and nonsurvivors ¹⁷¹. Whether the recruitment of circulating EPCs might have a beneficial effect on the clinical course in sepsis has still to be elucidated.

1.1.6 CYTOKINE-INDUCED SIGNALING IN ENDOTHELIAL CELLS

TNF- α induced signaling pathways

Several aspects of the interaction between host and pathogen must be considered in drawing a picture of the immune events that underlie the cause of sepsis. The initial activation of innate immunity by, e.g., LPS, may lead to release of e.g., TNF- α , IL-1, IL-6 and HMGB-1. The molecular changes underlying cellular dysfunction are hence a likely result of cytokine activation.

The signaling pathways in endothelial cells induced by pro-inflammatory cytokines comprehend a complex system ¹⁷². As in most other cells in the body, TNF- α -induced effects are mediated by TNFR1 ¹⁷³. Upon binding of TNF- α to TNFR1 on the endothelial cells, multiple pathways are activated leading to an inflammatory status of the endothelial cell (fig. 9) ¹⁷⁴. TNFR1 activation provides a docking site for accessory proteins that form the branching point for the pro-inflammatory and pro-apoptotic signaling pathways, eventually leading to the activation of the transcription factor nuclear factor κ B (NF κ B), a dimer usually consisting of p65 (RelA) and p50 (NF κ B1) ^{175, 176}. In this cascade NF κ B-inducing kinase NIK activates inhibitor of κ B (I κ B) kinases IKK α and IKK β . IKKs form a catalytic subunit responsible for phosphorylation, site-specific ubiquitinylation and subsequent proteasomal degradation of I κ B. Upon degradation of I κ B, the nuclear localization sequence of NF κ B targets the protein into the nucleus and binds to DNA. By interaction with co-activators and other components of the gene transcription machinery, NF κ B activates transcription of a range of inflammatory genes. Besides being controlled by IKK activity, reactive oxygen species prominently affect NF κ B activity ¹⁷⁷. Following TNF- α induced NF κ B activation, endothelial cells express functionally related genes, including the pro-inflammatory adhesion molecules E-selectin, ICAM-1, and VCAM-1, cytokines IL-6 and IL-8, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) ^{173, 178}. In parallel, genes involved in controlling cell signaling and apoptosis/cell proliferation are down-regulated ¹⁷⁹. NF κ B dependent transcription is only transiently activated upon stimulation due to feedback mechanisms including the induction of I κ B gene transcription and translation by NF κ B, and IKK β autophosphorylation in its inhibitory loop ¹⁸⁰.

Besides inducing a pro-inflammatory status, NF κ B signaling also leads to the expression of protective genes, such as zinc finger protein A20 ¹⁸¹, bcl-related gene A1 ¹⁸¹ and inducible hemeoxygenase-1 (HO-1) ¹⁸². It is assumed that these protective genes prevent the endothelium from going into apoptosis upon activation. At the same time, they down-regulate the endothelial pro-inflammatory response.

Upon TNF- α activation also mitogen-activated protein kinase (MAPK) become activated in endothelium. The 3 major MAPK signaling pathways comprise the extracellular-regulated protein kinase (ERK) pathway, the c-Jun NH2-terminal kinase (JNK) pathway, and the p38 MAPK pathway. They are involved in a network of signaling routes leading to cell proliferation, transformation and differentiation (ERK regulated), and apoptosis, stress responses and inflammation (JNK and p38 MAPK regulated) ^{183, 184}. Of the MAPK-family, p38 MAPK is involved in TNF- α -driven IKK activation ¹⁸⁵. Furthermore, p38 MAPK can control mRNA stability of inducible cytokines TNF- α , IL-1 and IL-8. JNK activation in HUVEC resulted in ICAM-1 and VCAM-1 expression ¹⁸⁶.

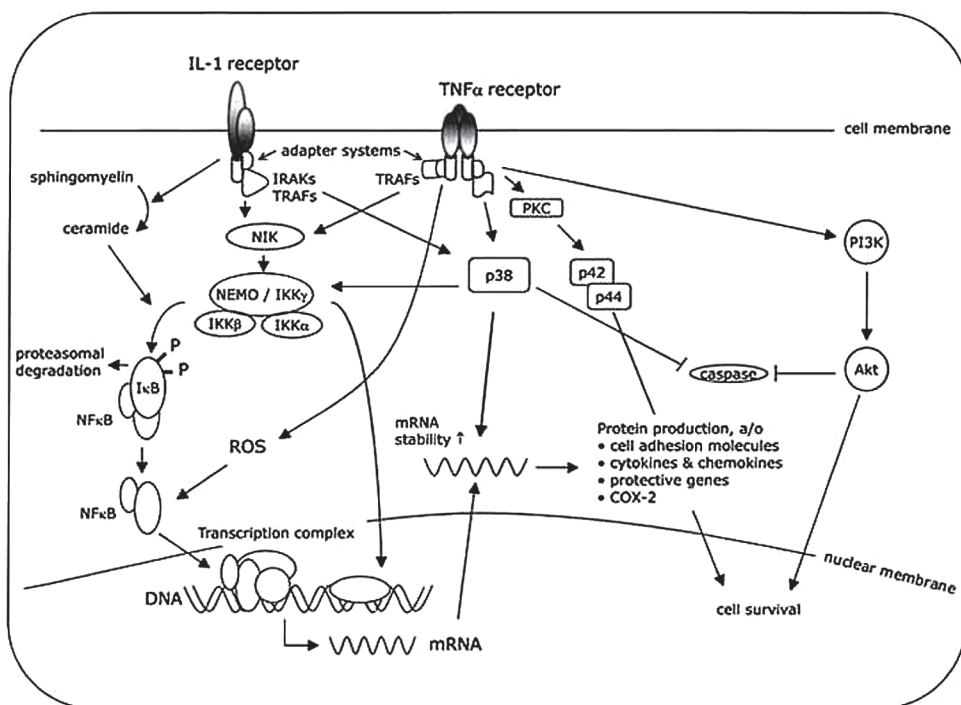


Figure 9. Simplified representation of the signaling pathways that become active in endothelial cells upon interleukin-1 (IL-1) and tumor necrosis factor (TNF)- α activation. The activation leads to the expression of pro-inflammatory genes, including cell adhesion molecules, cytokines and chemokines, and cell survival genes. Abbreviations: IKK: I κ B kinase; I κ B: inhibitor κ B; IRAK: IL-1 receptor-1 associated kinase; NEMO: NF κ B essential modulator; NF κ B: nuclear factor κ B; NIK: NF κ B inducing kinase; PI3K: phosphoinositol-3 kinase; PKC: protein kinase C; ROS: reactive oxygen species. Reprinted with permission from Kuldo JM, Ogawara KI, Werner N, Asgeirsdóttir SA, Kamps JA, Kok RJ, Molema G. (2005) Molecular pathways of endothelial cell activation for (targeted) pharmacological intervention of chronic inflammatory diseases. *Curr Vasc Pharmacol*;3:11-39. © 2005 Bentham Science Publishers Ltd.

LPS-induced signaling pathways

Most pathogens are detected by the innate immune system via Toll-like receptors (TLRs). Currently, 13 different mammalian TLRs have been identified¹⁸⁷. TLRs are pattern recognition receptors, which recognize PAMPs. PAMPs are small molecular sequences unique to microorganisms that enable the host to recognize foreign pathogens¹⁸⁸. Toll-like receptors 1, 2, 4, 5 and 6 are expressed on the cell surface, where they specialize in the recognition of bacterial products, including bacterial lipoproteins and lipoteichoic acids (TLR-2) or LPS (TLR-4), which are unique to the cells walls of Gram-

positive and Gram-negative bacteria. In fact, bacterial components signal via a single TLR, but studies have shown that cell wall extracts from Gram-positive and Gram-negative organisms contain components that can activate both receptors^{189,190}. However, mice deficient in TLR-2 are more prone to infection with staphylococci¹⁹¹ and TLR-4 deficient mice are more prone to infections with *Salmonella* spp.¹⁹². This suggests that Gram-positive infections have a TLR-2-dominant and Gram-negative infections a TLR-4-dominant signal.

LPS cell interaction involves the binding to LPS-binding protein (LBP) that transfers LPS to CD14. CD14 increases the LPS responses, but is not specific for LPS, because it enhances also immune responses to other pathogen products¹⁹³. In addition, LPS responses are not always dependent on CD14¹⁹⁴. Formation of the complex between LPS and CD14 facilitates the transfer of LPS to the LPS receptor complex composed of TLR-4 and MD-2¹⁹⁵. The TLR-4/MD-2 complex signals through adaptor molecules, myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain containing adaptor protein (TIRAP), Toll/IL-1 receptor domain containing adaptor inducing interferon- β (TRIF), and TRIF-related adaptor molecule (TRAM). These constitute two signaling pathways, one is MyD88-dependent and requires TIRAP, and the other is TRIF-dependent and requires TRAM (fig. 10)¹⁹⁶⁻¹⁹⁸. Both adaptor proteins initiate a signaling cascade culminating in the activation of the transcription factors NF- κ B, activator protein 1 (AP-1), and interferon regulatory factors (IRFs), subsequently leading to the induction of several inflammatory genes¹⁹⁹.

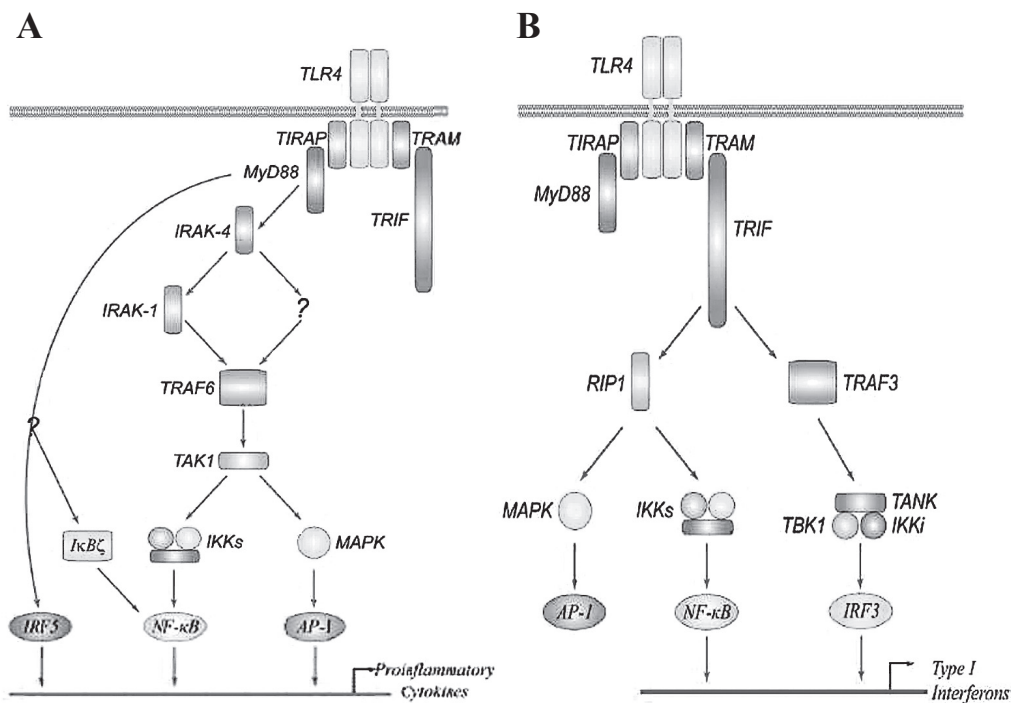


Figure 10. Toll-like receptor (TLR) 4 signaling pathway. (A) The MyD88-dependent pathway. MyD88 activates IRAKs/ TRAF6 as well as the transcription factors NF- κ B, AP-1 and IRF-5 further downstream. These transcription factors induce expression of pro-inflammatory cytokine genes. (B) The MyD88-independent pathway. TRIF signals the induction of Type I interferons by recruiting TRAF3 and RIP1 to activate transcription factor IRF3, as well as NF- κ B and AP-1. Reprinted with permission from Lu YC, Yeh WC, Ohashi PS. (2008) LPS/TLR4 signal transduction pathway. *Cytokine*;42(2):145-51. © 2008 Elsevier Ltd.

In clinical studies, an enhanced TLR-2 and TLR-4 expression was observed in leukocytes from septic patients compared with healthy controls²⁰⁰. In contrast, patients with septic shock were found to have decreased expression of TLR-2 and a trend to decreased expression of TLR-4²⁰¹. These findings corresponds to the differing inflammatory responses in the clinical stages of sepsis with an up-regulation (sepsis) or down-regulation (severe sepsis/septic shock) of inflammatory cytokine production. Salomao et al. used polymerase chain reaction-array to evaluate the gene expression of genes related to TLR-mediated signal transduction in patients with sepsis, severe sepsis, and septic shock. The genes investigated included genes encoding for TLRs, adaptor

and interacting proteins, effectors, downstream pathways and target genes (NFκB, JNK/p38 pathway and IRF pathway). Their results showed that TLR-signaling pathway genes are differently regulated in peripheral blood mononuclear cells and neutrophils of septic patients, and that they are dynamically modulated across the different stages of sepsis²⁰². The gene expression of PBMCs revealed that the major differences between septic shock patients and healthy volunteers consisted of a down-regulation of genes in the septic shock group, mostly from the NFκB and JNK/p38 pathway. In contrast, the changes in gene expression observed in neutrophils comprised predominantly an up-regulation of all genes groups evaluated which persisted along the clinical spectrum of sepsis.

Evaluation of TLR expression and mapping of the intracellular signaling pathways are important aspects to understand the cell adaptation in their complex functions of sensors and effectors of host responses during sepsis.

The role of NFκB in sepsis

Several lines of evidence indicate a role of NFκB activation in the pathophysiology of sepsis, since a variety of pathogens known to cause sepsis and pro-inflammatory cytokine release during sepsis can activate NFκB²⁰³. In peripheral mononuclear cells from septic patients NFκB activity is markedly increased, and the level of NFκB activity correlates with disease severity^{204, 205}. In animal models of septic shock induced either by LPS or by CLP, NFκB inhibitors protected animals from septic lethality²⁰⁶⁻²⁰⁹. Also, molecules proven to protect mice from lethal endotoxemia, such as IL-10²¹⁰, or to improve survival in severe sepsis patients, such as activated protein C²¹¹, exert their protective effect by inhibiting NFκB activation^{212, 213}. In models of endotoxin tolerance, cells or animals exhibited a down-regulated NFκB activity and reduced expression of NFκB-dependent genes, when subsequently exposed to endotoxin²¹⁴.

In the pathophysiology of sepsis transcriptional activation of multiple inflammatory genes marks an important characteristic. Studies have demonstrated that NFκB plays a crucial role in LPS- or cytokine-activated promoter activity of over 200 genes, many of which play important roles in the development of septic shock^{178, 213, 215}. These genes include cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines (e.g., IL-8), adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin, P-selectin), enzymes (e.g., iNOS, COX-2) and acute-phase-proteins^{178, 213, 215}. *In vivo* inhibition of NFκB activation in animal models demonstrated reduced LPS-induced mRNA and protein expression of multiple pro-inflammatory cytokines and other molecules that play critical roles in the pathophysiology of sepsis^{213, 216-218}.

Also, amelioration of the vascular derangement in both LPS and CLP models of septic shock could be demonstrated by inhibition of the NFκB pathway, which resulted in restored systemic hypotension^{209, 218}, reversal of the depressed vascular contractile response²¹⁹ and restoration of the impaired endothelium-dependent vasodilator

response^{208, 220}. Furthermore, NFκB is involved in multiple steps of the coagulation cascade^{178, 221-223}, which shows abnormalities in sepsis including DIC. Inhibition of NFκB activation prevents coagulation, resulting in improved outcome of septic shock²⁰⁴.

The critical role of NFκB activation in septic pathophysiology and the effectiveness of inhibiting NFκB activation in correcting septic abnormalities indicate that targeting NFκB is a potential therapeutic strategy for the treatment of septic shock. However, NFκB activation controls not only the inflammatory response in the pathophysiology of sepsis, but also the bacterial clearance and normal cell homeostasis. Disruption of the NFκB signaling pathway impairs the host defense capacity to eliminate invading bacteria and leads to a worsened outcome in a bacterial infection model of sepsis²²⁴⁻²²⁷, indicating that NFκB is also protective. NFκB inhibitors are not capable of differentiating between these processes so far. To be able to use inhibition of NFκB activation as an effective therapy option, strategies have to be developed to inhibit NFκB activation without interfering with the host-defense functions.

Just recently, an interesting study was published investigating the role of endothelial-intrinsic NFκB activity in multiple organ injury and host defense in response to sepsis using both LPS and CLP models of sepsis²²⁸. To this end, double transgenic (TG) mice were generated that conditionally overexpressed a degradation-resistant form of IκBα (IκBamt), a superior inhibitor of NFκB, selectively on endothelium. Until now, investigations into the role of NFκB activation in sepsis and other inflammatory conditions have been hampered by the fact that NFκB knockout mice are embryonically lethal^{224, 229-232}. Also, conventional TG mice overexpressing IκBamt selectively in endothelium were not qualified, since they have structural and functional defects in endothelium, as indicated by loss of endothelial tight junction, increased sensitivity to LPS-induced endothelial permeability, and enhanced susceptibility to tumor metastasis²³³. Ye et al.²²⁸ overcame these problems by taking a conditional TG approach using a tetracycline-regulated gene expression system. Therefore, the mice did not express IκBamt until induced by feeding with Dox, and had normal NFκB activity that is critically required for embryonic development. The NFκB inhibition was transient and restricted to endothelium, which had minimal effects on immune cell differentiation, development and function allowing the study of septic response under a physiological setting. When subjected to endotoxemia, TG mice showed endothelial-selective blockade of NFκB activation, repressed expression of multiple endothelial adhesion molecules, reduced neutrophil infiltration into multiple organs, decreased endothelial permeability, ameliorated multiple organ injury, reduced systemic hypotension, and abrogated intravascular coagulation. The TG mice also exhibited alleviated multiple organ injury and improved survival in the CLP sepsis model compared with wild-type (WT) mice²²⁸. Thus, selective blockade of the endothelial NFκB pathway is sufficient to reduce multiple organ inflammation, prevent organ injury and improve survival, indicating that endothelial NFκB plays a critical role in septic multiple organ inflammation and

injury. The study also demonstrated that WT and TG mice had comparable capacity to clear three different pathogenic bacteria, *S. pneumoniae*, *L. monocytogenes* and *S. enterica* ²²⁸, indicating that endothelial NFκB does not play an important role in the host defense response to eliminate those pathogenic bacteria. This can be explained by a different host defense mechanism involved in the clearance of the three pathogenic bacteria ²³⁴. Since NFκB activation is a key component of host immune response ²²⁷, and NFκB p50 knockout mice showed severely defective clearance of *S. pneumoniae* and *L. monocytogenes* ²²⁴, the lack of effect of endothelial-selective NFκB inhibition on bacterial clearance is surprising. Studies have to be conducted to investigate which cellular NFκB system plays a major role in the host defense response against bacterial pathogens. In summary, these results demonstrate that endothelial NFκB plays divergent roles in the inflammatory and host defense responses against bacterial infection. Therapeutical strategies have to be developed to selectively inhibit the endothelial NFκB pathway.

1.1.7 THE ROLE OF CARBON MONOXIDE IN SEPSIS

As described in chapter 1.1.2, the inflammatory process involves a multitude of mediators and systems. Also, the heme oxygenase system (HO) has been demonstrated to be involved in the control of inflammatory processes ²³⁵⁻²³⁸, in addition to its role in oxidant-induced injury ²³⁹⁻²⁴¹. HOs are the rate-limiting enzymes in degradation of heme into carbon monoxide (CO), Fe²⁺ and biliverdin, the latter being subsequently converted to bilirubin ²⁴². The HO system comprises several isoenzymes ^{243, 244}, of which the inducible HO-1 isoenzyme is particularly important as an anti-inflammatory mediator ^{235-237, 245}. Studies in HO-1-null mice and reports on human HO-1-deficiency have strengthened the evidence that HO-1 is an important molecule in host defense against oxidant stress, and have also emphasized the potent anti-inflammatory properties of HO-1 ^{245, 246}. In these studies both mice and humans deficient in HO-1 expression showed increased vulnerability to inflammation. Furthermore, exogenous administration of HO-1 by gene transfer into rat lung provided protection against hyperoxia-induced lung injury *in vivo* by modulation of neutrophil inflammation and lung apoptosis ²⁴⁷.

The protective function of HO-1 has been attributed to several possible mechanisms. All three HO-reaction products (*i.e.*, iron, biliverdin, and CO) have been extensively discussed as potentially contributing to HO-mediated cytoprotection and anti-inflammatory mediators. However, some of the published data are controversial. A number of studies describe a down-regulation of VCAM-1 and E-selectin expression by HO-1 via bilirubin and iron chelation with no apparent involvement of CO in HUVEC ^{236, 237}. Others clearly demonstrate the anti-inflammatory potential of HO-1 mediated CO production in macrophages and monocytes ²³⁵ as well as in rat pulmonary artery endothelial cells ^{248, 249}. The salutary effect of CO has also been shown for organ

transplantation and ischemia reperfusion injury animal models ^{250, 251}. The increase in inflammatory mediators was markedly inhibited in CO-treated recipients compared to non-treated recipients, and the inhibition correlated with improved renal cortical blood flow. When looking at pro-inflammatory cytokines, CO significantly inhibited LPS-induced TNF- α production, but it did not completely prevent the production of this cytokine by monocytes *in vitro* and *in vivo* ²³⁵.

Carbon monoxide

Carbon monoxide (CO) is a low-molecular-weight gas molecule that arises in nature as the product of the combustion of organic matter, such as from the burning of fossil fuels or tobacco ²⁵². Environmental CO represents a major air pollutant, which is generally regarded as an inhalation hazard. It is well known that high inspired concentrations of this gas are toxic. The binding of CO to hemoglobin (Hb) inhibits O₂ transport and delivery to tissues. Thus, at elevated concentrations, CO acts as an asphyxiant, which causes tissue hypoxia that is associated with a number of clinical symptoms, including dizziness, loss of consciousness, and death upon prolonged or excessive exposure ^{252, 253}. Symptoms of CO poisoning in humans appear at carboxyhemoglobin (CO-Hb) levels of 20%, whereas loss of consciousness (coma) leading to death occurs in the range of 50–80% CO-Hb ²⁵².

However, CO has recently emerged as a potential therapy for sepsis ²⁵⁴, based on its vasodilatory, anti-ischemic and anti-inflammatory activities ²⁵⁵. Observations of dramatic tissue protection from the application of low concentrations of CO in animal models of inflammation, sepsis, oxidative stress, and ischemia/reperfusion injury raised the possibility to use this gas clinically ^{253, 256}.

Dosage and application form

The dose of CO that has been applied experimentally in animal studies ranges from 10 to 1,000 parts per million (ppm). Most investigators have applied CO by inhalation of 250 ppm, corresponding to an inspiratory fraction of 0.025%. CO diffuses from the alveolar space into the capillaries and subsequently binds Hb for transport to the different tissues in the body. The extent of CO-Hb formation depends on the dose and the time of application ²⁵⁴. For cell culture experiments, also 5% CO₂ was present for buffering requirements.

As an alternative to inhalation for delivery of CO, molecules have recently been developed that are composed of transition metal carbonyls and capable of liberating CO in dose- and time-dependent fashion. These are referred to as carbon monoxide-releasing molecules (CORMs) ^{255, 257, 258}. Water-soluble forms of CORMs allow intravenous administration. In particular, CORM-3 (tricarbonylchloro(glyconato)ruthenium(II)) and CORM-A1 (sodium boranocarbonate),

which are both fully water-soluble, rapidly liberate CO when dissolved in physiological solutions ^{259, 260}. Interestingly, and in contrast to inhaled CO, CORMs appear to deliver CO directly to the tissues without significant formation of CO-Hb ²⁶¹. These molecules might therefore, besides serving as research tools, also be of therapeutically interest to modulate ongoing inflammatory reactions by delivering CO in a controllable fashion ²⁶².

The role of CO in sepsis

Sepsis can affect the synthesis of CO in humans. HO-1 expression and consequent CO production are up-regulated in aortic smooth muscle cells and polymorphonuclear cells of septic patients ²⁶³. Furthermore, the levels of CO measured on the exhaled breath were higher in patients suffering from severe sepsis as compared with critically ill patients without sepsis ²⁶⁴. Sepsis survivors in the same study showed higher levels of exhaled CO than nonsurvivors. Similar findings were demonstrated for pediatric patients. CO-Hb levels in the blood of septic newborn infants ²⁶⁵ and septic children ²⁶⁶ were elevated and more pronounced in septic shock compared to healthy controls. Based on these observations, it was hypothesized that up-regulation of endogenous CO synthesis might act as a protective mechanism mediating the anti-inflammatory response under septic conditions. Many studies have been conducted to investigate whether exogenous CO application might improve outcome and survival in animal models of sepsis. These studies demonstrated a protective effect of CO administration associated with prolonged survival and reduced mortality ^{235, 267-270}. The exact mechanisms by which CO exerts these effect are still unknown, yet multiple effects on the inflammatory response have been proposed.

Anti-inflammatory effects of CO in sepsis

CO can exert direct effects on immune competent cells, such as inhibiting the activation of monocytes, macrophages and leukocytes *in vitro* and *in vivo* ^{268, 271, 272}. Furthermore, it suppresses the ability of T-cells to proliferate ²⁷³, and reduces the adhesion and migration of leukocytes in rats ²⁷⁴.

In vitro, CO inhibits the LPS-induced production of pro-inflammatory cytokines such as TNF- α , IL1- β and macrophage inflammatory protein-1 β (MIP-1 β) in cultured macrophages ²³⁵. Also, the application of CORM (*e.g.*, CORM-3) has been demonstrated to reduce the level of TNF- α in murine macrophages ²⁷⁵. Furthermore, CO treatment promoted an increased production of the anti-inflammatory cytokine IL-10 during LPS challenge in cultured macrophages ²³⁵. Interestingly, the anti-inflammatory effect of IL-10 itself appeared to be mediated by HO activity and specifically required CO ²⁷⁶.

In vivo, these general anti-inflammatory effects of CO could also be observed. In murine endotoxemia models, CO preconditioning resulted in reduced production of serum TNF- α , IL-1 β , and IL-6, reduced organ injury and prolonged survival after LPS challenge ^{235, 269, 270}. And in line with the *in vitro*-studies, CO also increased dose-

dependently LPS-inducible IL-10 production ²³⁵. In a pig study, CO reduced the development of DIC and completely suppressed serum LPS-induced IL-1 β levels, while amplifying LPS-induced IL-10 production ²⁷⁷. IL-10 is not absolutely essential for the anti-inflammatory effect of CO as in IL-10^{-/-} mice, CO inhibited LPS-induced TNF- α levels within the first hour to a similar extent as in wild-type mice ²³⁵.

The application of CO does not only affect cytokine release, but also the activation of MAPK. In RAW 264.7 murine macrophages, CO application reduced the production of LPS-induced cytokine release on one side, and increased p38 MAPK activation on the other side. Of the MAP kinase kinases (MKK3, MKK4, and MKK6) that activate p38 MAPK, CO enhanced the LPS-mediated stimulation of MKK3 in RAW 264.7 and epithelial cells ^{235, 278-280}. In contrast, the CO-triggered down-regulation of LPS-induced IL-6 production in macrophages was mediated via the JNK-pathway, which regulates several transcription factors including AP-1 ²⁶⁹. The IL-17-induced IL-6 production in pulmonary epithelial cells on the other hand, was inhibited by CO *via* the ERK1/2-dependent pathway without altering p38 MAPK or JNK ²⁸¹. In rat pulmonary artery endothelial cells treated with TNF- α , the exposure to CO specifically reduced ERK1/2 activation and increased p38 MAPK activation ²⁴⁸. Taken together, CO exerts differential effects on p38 MAPK, JNK, and ERK1/2, which are dependent on cell type and stimulus. *In vivo*, the responsiveness of TNF- α to LPS treatment, as well as the inhibitory effects of CO, appeared down-regulated in MKK3^{-/-} mice compared with wild-type mice, suggesting that the MKK3/p38 MAPK pathway might play an important role in CO-mediated anti-inflammatory signaling ²³⁵.

Downstream of p38 MAPK, heat-shock proteins appear to play an important contributory role in the anti-inflammatory effects of CO. Increased expression of heat-shock protein 70 (Hsp70) was involved in the protective effects of CO in murine lung endothelial cells and fibroblasts ²⁸². Suppression of Hsp70 expression and/or genetic deletion of heat shock factor-1, the principle transcriptional regulator of Hsp70, attenuated the cytoprotective and immuno-modulatory effects of CO in mouse lung cells and *in vivo*. Recently, it was demonstrated in a CLP-model that HO-1-derived CO affects the antimicrobial process without inhibiting the inflammatory response ²⁸³. Mice overexpressing HO-1 in smooth muscle cells (SMC) of blood vessels and bowel ameliorated sepsis-induced death associated with *Enterococcus faecalis* infection ²⁸³. However, the increase in HO-1 expression did not suppress circulating inflammatory cells or their accumulation at the site of injury, but did enhance bacterial clearance by increasing phagocytosis and the endogenous antimicrobial response ²⁸³. Furthermore, injection of CORM-3 into WT mice increased phagocytosis and rescued HO-1-deficient mice from sepsis-induced lethality ²⁸³.

In an initial attempt to translate the anti-inflammatory effects of CO observed in rodents to humans, a preclinical trial was performed ²⁸⁴. Humans were exposed to 500-ppm CO for 1 h by inhalation, a dose that increased CO-Hb levels to 7%, followed by LPS

injection. LPS infusion transiently increased plasma concentrations of TNF- α , IL-6, and IL-8, as well as IL-1 α and IL-1 β mRNA levels. CO inhalation did not influence the cytokine response to endotoxin treatment in humans ²⁸⁴. Since many sepsis models have shown protective characteristics of CO application, further studies with different designs have to be conducted to investigate the potential of CO for reducing inflammation in septic patients.

1.2 AIM OF THE THESIS

As summarized in Chapter 1.1, sepsis is a complex clinical syndrome and comprises multiple derangements involving several different organs and physiological systems. There is convincing evidence that in addition to monocytes, also endothelial cells play an important role in the clinical outcome of sepsis, but the actual role of the endothelium in the course of sepsis is not fully understood.

In this thesis we aimed to gain insight in the heterogenous response of macro- and microvascular endothelial cells to inflammatory stimuli, to study the anti-inflammatory effect of the signaling molecule carbon monoxide (CO) and the mechanisms involved, and to investigate whether bone marrow-derived endothelial progenitor cells are increasingly mobilized in the course of sepsis and if this mobilization is associated with clinical outcome.

In **Chapter 2** we tested the hypothesis that the response of endothelial cells to lipopolysaccharide (LPS) can be classified into general phenotypes of cells with a low and a high pro-inflammatory potential. Based on previous findings that serum IL-8 concentration is associated with severity of sepsis, and the fact that endothelial cells are the main origin of IL-8, endothelial cells were grouped according to their IL-8 production. We investigated by gene expression profiling whether low IL-8 production is associated with low expression of other inflammatory genes and which molecular mechanism might be involved in this phenomenon.

In **Chapter 3** we studied whether high concentrations of CO releasing molecules (CORM-3), which release CO in excess of endogenously produced CO, are able to modulate the expression of adhesion molecules by endothelial cells, and if this was mediated by similar mechanisms as have been reported for heme oxygenase (HO)-1. In **Chapter 4** we set out to investigate the molecular mechanism involved in the CO-mediated down-regulation of VCAM-1 on endothelial cells.

In **Chapter 5** we tested the hypothesis that clinical outcome in septic patients is largely dependent on the ability to reconstitute damaged endothelium. We used flow cytometry to identify and detect circulating endothelial progenitor cells in the peripheral blood of septic patients and studied whether this mobilization was associated with clinical outcome.

Chapter 6 provides a summary of the generated data and discusses some important issues raised during this research, highlighting future perspectives of potential therapeutic interventions, including CO-mediated interference with activation of

endothelial cells in sepsis and application of endothelial progenitor cells to maintain the integrity of the endothelial layer.

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